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14. ABSTRACT
Despite advances in the early detection and treatment of breast cancer, the mortality rate for 20% of patients with recurrences and/or metastases is nearly 100% (1). Thus, it is critical to understand the mechanism of breast cancer metastasis to allow for better therapies targeted at the cellular culprits of metastasis. The current dogma of metastasis is that most primary tumor cells have low metastatic potential, but rare cells, less than one in ten million, within large primary tumors acquire metastatic capacity through somatic mutation. The metastatic phenotype includes the ability to disseminate from the primary tumor, survive in blood or lymphatic circulation, invade distant tissues and establish macroscopic metastatic nodules. This dogma is primarily supported by animal models in which highly metastatic clones can develop from poorly metastatic cell lines if the process is facilitated by the isolation of metastatic nodules, expansion of the cells in vitro, and injection of these selected cells into recipient mice. However, no direct evidence of this genetic selection model has been documented in human tumors. A recent report demonstrated that a subpopulation of breast tumor cells (CD44+/CD24-(low)/Lineage-) isolated from breast cancer patient samples, even as few as 200 cells, were able to give rise to bulky tumors, greater than 1 cm in diameter, in NOD/SCID mice. Moreover, this discrete cell population has the ability to proliferate extensively, and to give rise to diverse and more differentiated cell types with reduced developmental or proliferative potential suggesting that these highly tumorigenic CD44+/CD24-(low)/Lineage- cells may, indeed, be breast tumor stem cells. With this information, we propose an alternate model of metastasis and hypothesize that the breast tumor stem cells are the subpopulation of cells that are present in the heterogeneous primary breast tumor and possess the unique properties of an angiogenic and metastatic phenotype.

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Table of Contents

Introduction……..4

Body……4

Key Research Accomplishments……7

Reportable Outcomes……7

Conclusions……7

References……7

Introduction:

Despite advances in the early detection and treatment of breast cancer, the mortality rate for 20% of patients with recurrences and/or metastases is nearly 100% (1). Thus, it is critical to understand the mechanism of breast cancer metastasis to allow for better therapies targeted at the cellular culprits of metastasis. The current dogma of metastasis is that most primary tumor cells have low metastatic potential, but rare cells, less than one in ten million, within large primary tumors acquire metastatic capacity through somatic mutation (2). The metastatic phenotype includes the ability to disseminate from the primary tumor, survive in blood or lymphatic circulation, invade distant tissues and establish macroscopic metastatic nodules. This dogma is primarily supported by animal models in which highly metastatic clones can develop from poorly metastatic cell lines if the process is facilitated by the isolation of metastatic nodules, expansion of the cells *in vitro*, and injection of these selected cells into recipient mice (3). However, no direct evidence of this genetic selection model has been documented in human tumors. A recent report demonstrated that a subpopulation of breast tumor cells (CD44+/CD24-(low)/Lineage-) isolated from breast cancer patient samples, even as few as 200 cells, were able to give rise to bulky tumors, greater than 1 cm in diameter, in NOD/SCID mice (4). Moreover, this discrete cell population has the ability to proliferate extensively, and to give rise to diverse and more differentiated cell types with reduced developmental or proliferative potential suggesting that these highly tumorigenic CD44+/CD24-(low)/Lineage- cells may, indeed, be breast tumor stem cells (4). With this information, we propose an alternate model of metastasis and hypothesize that the breast tumor stem cells are the subpopulation of cells that are present in the heterogeneous primary breast tumor and possess the unique properties of an angiogenic and metastatic phenotype. This proposal will address the importance of tumor stem cells in breast cancer metastasis using an appropriate animal model. As a corollary, it may suggest therapies that selectively target the breast tumor stem cell population leading to abrogation of clinical metastasis, a recalcitrant challenge in breast cancer.

Body:

Specific Aim 1:

1. Determine the in vitro angiogenic potential of unsorted breast tumor cells, sorted putative stem tumor cells, and established breast cancer cell lines using the rat aortic ring, endothelial cell vessel formation, and endothelial cell migration assay (Months 1-4).

Unsorted Her/neu murine tumor cells, CD44+/CD24-, and CD44-/CD24+ cells were plated out and conditioned-media was collected. We perform ELISA assays for VEGF, a well-recognized player in angiogenesis, and did not see a statistically difference between the groups (Figure 1). We did not observe consistent induction of endothelial cell vessel formation or endothelial cell migration assay with unsorted, CD44+/CD24-, and CD44-/CD24+ cells. Results using conditioned-media from breast cancer cell lines worked as expected, thus we do not think this is a technical issue. We believe that this may be due to the fact that we were using human endothelial cells and not murine endothelial cells. In any event, our result with VEGF indicated that the angiogenic potential of putative tumor stem cells was similar to nonputative tumor stem cells.

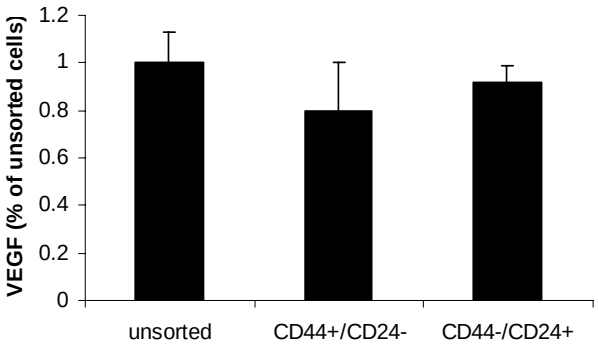

Figure 1. VEGF levels of putative tumor stem cells (CD44+/CD24-) is similar to nonputative tumor stem cells (CD44-/CD24+). Conditioned-media from unsorted Her2/neu tumor cells, CD44+/CD24-, and CD44-/CD24+ cells were collected. VEGF levels were measured by ELISA. p-value > 0.05

2. Determine the in vitro invasion and motile potential of unsorted breast tumor cells, sorted putative stem tumor cells, and established breast cancer cell lines (Months 3-6).

Unsorted Her/neu murine tumor cells, CD44+/CD24-, and CD44-/CD24+ cells were assessed for invasion using the standard Matrigel invasion-chamber assay. As shown in Figure 2, putative stem cells were 45-50% more invasive than unsorted or nonputative stem cells; however, this difference did not reach statistically significance ($p > 0.05$) using the two-tailed Student's t-test.

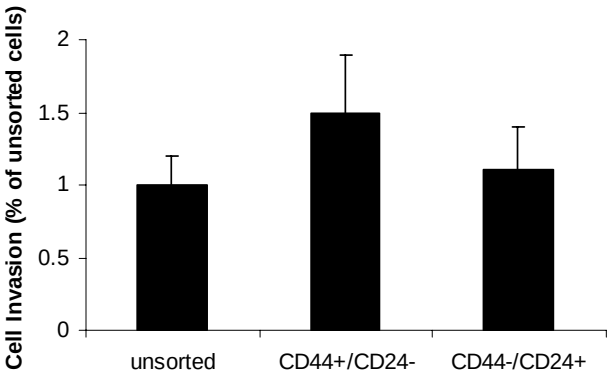

Figure 2. Cell invasion of putative stem cells (CD44+/CD24-) is similar to nonputative stem cells (CD44-/CD24+). Unsorted Her2/neu tumor cells, CD44+/CD24-, and CD44-/CD24+ cells were assessed for cell invasion using the Matrigel invasion-chamber assay. p-value > 0.05

Specific Aim 2:

1. Determine the in vivo metastasis potential of unsorted breast tumor cells and sorted putative stem tumor cells using the tail vein injection pulmonary colonization and intracardiac injection bone metastasis models (Months 6-9).
2. Isolate tumor cells from the metastases, resort for putative breast tumor stem cells, and serially transplant into secondary recipient mice. Determine the in vivo metastatic potential of serially transplanted putative breast tumor cells (Months 7-12).
3. Characterize the heterogeneity of the tumor cells from the metastases using flow cytometry (Months 9-12).

We were successful in enriching putative breast tumor stem cells from tumor in Her2/neu transgenic mice using two cell surface markers, CD44 and CD24. Sorted, CD44+/CD24- cells were found to be more tumorigenic than sorted, CD44-/CD24+ cells. However, the results were not as clear cut as we wanted. CD44+/CD24- cells did not consistently form tumors and moreover, CD44-/CD24+ cells were able to form tumors in some cases. It is clear from these results that although CD44+/CD24- cells were more tumorigenic than CD44-/CD24+ cells, the strict use of these two markers to identify putative breast tumor stem cells may not sufficient in this model. In any event, we went forward with the project and started with Aim 2.1. We injected unsorted, sorted CD44+/CD24-, or sorted CD44-/CD24+ (10,000) cells in the tail vein of athymic nude mice (10 animals per group). After 8 weeks, mice were euthanized and lungs were resected for analysis. Lungs were sectioned, H&E stained, and analyzed for the presence of tumor cells in the lungs. Lung sections from animals in all three groups were absent of tumor cells indicating that injected cells did not metastasize to the lungs. These negative results could be due to two reasons; the use of CD44 and CD24 may not be sufficient to adequately enrich for putative breast tumor stem cells and the athymic nude mice used in this study may not be immunocompromised enough for tumor cells to

establish and grow. Our future plan is to continue this work and identify a better set of cell surface markers to enrich for putative breast tumor stem cells from Her2/neu transgenic mice. Based on the negative resulted obtained in Aim 2.1, we could not proceed to Aims 2.2 and 2.3.

Key Research Accomplishments:

1. CD44+/C24- (putative tumor stem cells) have similar VEGF levels and invasive potential compared to CD44-/CD24+ (nonputative tumor stem cells).

2. The use of CD44 and CD24 as cell surface markers to enrich for putative breast tumor stem cells from tumor isolated from Her2/neu transgenic mice may not be sufficient.

3. CD44+/CD24- (putative tumor stem cells) cells were unable to metastasize to lungs using the tail-vein pulmonary colonization metastasis model.

Reportable Outcomes:

No reportable outcomes.

Conclusions:

Our preliminary results suggest that the use of CD44 and CD24 to enrich for putative breast tumor stem cells from Her2/neu transgenic mice may not be sufficient. Further work with additional cell surface markers is needed to adequately identify putative breast tumor stem cells from Her2/neu transgenic mice.

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